EXHIBIT 4

RESEARCH LETTER

Assessment of SARS-CoV-2 RNA Test Results Among Patients Who Recovered From COVID-19 With Prior Negative Results

Some patients who have recovered from coronavirus disease 2019 (COVID-19) with documented negative real-time polymerase chain reaction (RT-PCR) results at the time of recovery have had subsequent positive RT-PCR test results for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1,2] in the absence of any symptoms suggestive of new infection.[3] It is unknown whether such patients are infectious and whether they should be quarantined. Real-time PCR is not a viral culture and does not allow determination of whether the virus is viable and transmissible. We investigated RT-PCR retested positive nasal/oropharyngeal swab (NOS) samples from recovered patients with COVID-19 with prior negative results for the presence of replicative SARS-CoV-2 RNA.[4]

Editor's Note

Supplemental content

Table. Testing Results for NOS Samples Obtained at COVID-19 Diagnosis or After COVID-19 Recovery in 32 Study Patients[a]

Sample	COVID-19 samples tested											Days of recovery sampling since diagnosis
	Diagnosis				Recovery							
	Genomic RNA (C_T value)			Subgenomic RNA (C_T value)	Genomic RNA (C_T value)			Subgenomic (C_T value)	RNA load, copies/mL	Serology (positive or negative result)		
Sample No.	*E* gene	*RdRP* gene	*N* gene	*E* gene	*E* gene	*RdRP* gene	*N* gene	*E* gene	*N* gene	IgG	IgA	
1	31.6	31.3	31.2	34.5	29.3	30.7	31.2	39.1	1.2×10^4	Positive	Positive	39
2	27.0	26.9	30.0	36.0	30.0	30.5	31.2		8.9×10^3	Positive	Positive	31
3	19.3	20.8	22.1	35.2	31.5	34.7	32.8		3.3×10^3	Positive	Negative	44
4	21.6	22.0	22.9	36.4	31.8	31.4	32.3		5.5×10^3	Positive	Positive	34
5	30.0	32.8	38.1	30.2	31.8	34.3	34.5		3.2×10^3	Positive	Positive	62
6	20.8	20.9	22.3	37.3	32.2	32.8	34.1		5.3×10^3	Positive	Positive	37
7	27.3	29.9	31.3	36.9	32.3	30.9	32.7		6.4×10^3	Positive	Positive	39
8	26.9	27.0	31.2	38.1	35.0	34.4	36.1		4.0×10^2	Positive	Positive	71
9	22.5	23.7	24.9	31.0	38.8	33.6	33.9		2.6×10^3	Negative	Negative	42
10	21.3	21.4	28.9	38.9		32.2	33.4		1.2×10^4	Positive	Positive	56
11	26.6	26.9	28.1	33.0		32.8	33.2		1.3×10^4	Positive	Positive	54
12	22.8	24.2	25.3	31.0		34.2	33.7		6.9×10^3	Positive	Positive	55
13	25.8	25.8	26.1	39.8	NA	34.8	39.1		3.0×10^2	Positive	Positive	36
14	20.8	20.4	21.1	32.0		35.0	35.1		1.9×10^3	Positive	Positive	56
15	29.4	30.1	32.2	37.0		36.5	39.2		3.2×10^3	Positive	Positive	36
16	27.9	29.1	31.1	32.0		38.1	39.3		1.6×10^1	Positive	Positive	77
17	30.6	29.9	31.8	32.1			35.7	NA	5.4×10^3	Positive	Positive	53
18	28.5	29.1	30.8	36.8			36.8		2.9×10^3	Positive	Positive	43
19	26.9	22.2	26.1	30.1			37.5		1.1×10^3	Positive	Positive	36
20	25.7	25.2	28.9	38.0			37.9		2.6×10^3	Positive	Positive	48
21	27.0	29.0	30.2	32.3			38.1		1.9×10^3	Positive	Positive	41
22	28.5	29.4	30.0	32.3			38.4		4.9×10^1	Positive	Negative	76
23	27.1	28.6	29.3	36.1			38.9		4.5×10^2	Positive	Positive	29
24	25.4	22.9	24.1	34.8			39.0		5.6×10^1	Positive	Positive	70
25	28.7	29.5	31.4	37.3	NA		39.1		5.4×10^3	Negative	Positive	46
26	27.1	27.7	29.2	37.1			39.1		1.9×10^3	Positive	Positive	34
27	26.7	27.7	29.6	39.2			39.2		2.0×10^3	Positive	Positive	45
28	17.1	19.1	19.9	33.0			39.2		8.5×10^2	Positive	Positive	40
29	27.0	28.9	30.0	32.1			39.3		5.0×10^1	Positive	Positive	56
30	22.9	23.8	25.8	37.1			39.4		1.6×10^2	Positive	Positive	55
31	28.6	30.4	30.9	33.0			39.6		5.3×10^2	Positive	Positive	61
32	29.1	28.0	30.9	36.2			39.8		3.4×10^2	Positive	Positive	53

Abbreviations: COVID-19, coronavirus disease 2019; C_T, cycle threshold; *E* gene, envelope gene; NA, not applicable; *N* gene, nucleocapsid gene; *RdRP*, RNA-dependent RNA polymerase; RT-PCR, real-time polymerase chain reaction.

[a] For RT-PCR testing, the Seegene Allplex 2019-nCoV and Clonit Quanty COVID-19 assays were used for total RNA detection and quantification, respectively, whereas replicative (*E* gene) RNA was detected by an in-house RT-PCR assay.[4] Results were expressed as C_T values (<40 for positive detection) or quantified as RNA (*N* gene) copies per mL. NA indicates the absence of positive detection for the indicated gene. For serological testing, SARS-CoV-2 IgG/IgA Euroimmun enzyme-linked immunoassays were used, and positive and negative results were assessed using the 1.1 or greater or less than 1.1 times the manufacturer's cutoffs as reference IgG/IgA values, respectively.

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Methods | We studied 176 recovered patients with COVID-19 who were admitted to the postacute outpatient service of our institution (Rome, Italy) from April 21 to June 18, 2020, for COVID-19 follow-up.[5,6] Before that, patients had discontinued isolation according to current criteria,[5] which require no fever for 3 consecutive days, improvement in other symptoms, and 2 negative RT-PCR results for SARS-CoV-2 RNA 24 hours apart.

Nasal/oropharyngeal swab samples from patients at follow-up were analyzed for total (genomic) and replicative (subgenomic) SARS-CoV-2 RNA using RT-PCR assays (eMethods in the Supplement). For patients with positive results for total RNA, samples previously obtained at the time of COVID-19 diagnosis and kept at −112 °F until testing were also tested for replicative RNA. Serological testing was performed for SARS-CoV-2 IgG/IgA detection (eMethods in the Supplement). The ethics committee of the Fondazione Policlinico Universitario A. Gemelli IRCCS (Rome, Italy) approved the study, and written informed consent was obtained from each patient.

Results | As shown in the **Table**,[4] 32 of 176 NOS samples (18.2%) tested positive for total SARS-CoV-2 RNA, with viral loads ranging from 1.6×10^1 to 1.3×10^4 SARS-CoV-2 RNA copies per mL. One of the 32 samples (3.1%) had replicative SARS-CoV-2 RNA. Samples from the 32 patients at the time of COVID-19 diagnosis were also tested and, expectedly, had replicative SARS-CoV-2 RNA. All but 1 of 32 patients had a positive serology result against SARS-CoV-2 (Table), as well as 139 of remaining 144 patients (data not shown), at COVID-19 follow-up. The patient who tested serologically negative was not the one with a positive test result for replicative SARS-CoV-2 RNA. The mean (SD) time from COVID-19 diagnosis to follow-up was 48.6 (13.1) days in 32 patients (Table) and 57.7 (16.9) days in 144 patients (data not shown).

Discussion | Similar to that reported elsewhere,[2] 18% of patients with COVID-19 in our institution became RT-PCR positive for SARS-CoV-2 RNA after clinical recovery and previous negative results.[5] As positivity in the patients was suggestive, but not necessarily a reflection, of viral carriage, we used replicative SARS-CoV-2 RNA detection as a proxy for virus replication in culture.[4]

Only 1 of 32 patients retesting positive had replicating virus in the NOS sample, suggesting either recurrent infection or reinfection, which is impossible to separate because no whole-genome sequencing and phylogenetic analyses were performed.[3] The patient retested positive 16 days after COVID-19 recovery (ie, 39 days from COVID-19 diagnosis) and was symptomatic. The patient was an older adult with hypertension, diabetes, and cardiovascular disease but no evidence of close contacts with people with SARS-CoV-2 infection or persons who became RT-PCR positive. In the 31 remaining patients (who were asymptomatic), their positive result likely represented either recurrent or resolving infection, but in either case, they were unlikely to be infectious. The limitations of our study are the lack of data from viral cultures or whole-genome sequencing analysis and the small sample size.

Conclusions | This study highlights that many patients who recovered from COVID-19 may be still positive (albeit at lower levels) for SARS-CoV-2 RNA, but only a minority of the patients may carry a replicating SARS-CoV-2 in the respiratory tract. Further studies are needed to verify whether such patients can transmit the virus.

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